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Taxonomy and Molecular Phylogeny of *Usnea rubicunda* and *U. rubrotincta* (*Parmeliaceae*, Lichenized *Ascomycotina*)

Yoshihito OHMURA

Department of Botany, National Museum of Nature and Science,
4-1-1, Amakubo, Tsukuba, Ibaraki, 305-0005 JAPAN
*E-mail: ohmura-y@kahaku.go.jp

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Usnea rubicunda and *U. rubrotincta* are distinguished by the mode of branch elongation, the amount of fibrils on branches and soralia morphology. Molecular phylogenetic analyses of these species with maximum parsimony, maximum likelihood and Bayesian methods based on internal transcribed spacer regions of nuclear ribosomal DNA (ITS rDNA) sequences support the monophyly of each species. Primers for the amplification of *Usnea* ITS rDNA sequence are newly designed.

Key words: Lichen, morphology, phylogeny, taxonomic characters, *Usnea*.

In the course of revisionary studies on the genus *Usnea* (*Parmeliaceae*, lichenized *Ascomycotina*), Ohmura (2001) reported *U. rubicunda* Stirt. and *U. rubrotincta* Stirt. as distinct species based mainly on examinations of Asian and Oceanian specimens including the related type specimens. These species share the following morphological characteristics: erect to subpendent thallus, and presence of reddish pigment in the cortex. However, they are distinguished by the mode of elongation of terminal and subterminal branches, the amount of fibrils on the branches, and soralia morphology and ontogeny. *Usnea rubicunda* has non-elongated terminal and subterminal branches with sparse fibrils. Soralia are absent or few on fibrils but abundant on thicker branches, and are rounded and stipitate on thicker branches. They seem to develop from the top of eroded papillae. While *U. rubrotincta* has elongated terminal and subterminal branches with abundant fibrils. Soralia are usually abundant on fibrils and other branches, and are punctiform to sinuose in shape on fibrils and lateral branches or rounded on thicker branches. They seem to develop from scars of detached fibrils or lateral branches.

The two species are chemically distinct from each other. *Usnea rubicunda* produces the stictic acid complex in the medulla whereas *U. rubrotincta* produces the salazinic acid complex. However, rare specimens of each taxon have been found to produce the chemistry of the other (Ohmura 2001).

Usnea rubicunda and *U. rubrotincta* were originally recognized by Stirton (1881) on the basis of chemical reactions in the medulla and axis [KOH (K) + yellow and iodine (I) + bluish for *U. rubicunda* and K + yellow to red and I – for *U. rubrotincta*; but Ohmura (2001) reported I – for both species]. Stirton (1883) described *U. rubescens* Stirt. in addition to these species. He recognized that it was similar to *U. rubrotincta* but distinguished by the pendulous or elongated thallus. Motyka (1936–1938) thought *U.*

rubrotincta and *U. rubescens* should be treated within the same species and he illegitimately placed *U. rubrotincta* under *U. rubescens* as a variety. Asahina (1953, 1956) basically followed Motyka's treatment (1936–1938), and he clearly mentioned the differences of branch elongation and fibril amount between the two species. Thus branch elongation has been considered to be a feature of *U. rubrotincta* but not *U. rubicunda*. Although several additional taxa were described according to chemical differences or the amount of red pigmentation in the cortex (Motyka 1936–1938, Asahina 1956, 1965, 1969, Stevens 1999), they were treated as synonyms of these species by Ohmura (2001).

While Ohmura (2001) recognized *U. rubicunda* and *U. rubrotincta* as distinct species, many authors have considered the differences of branch elongation, fibril amount, and soralia morphology in this group to fall within the variation of a single taxon, for which the name *U. rubicunda* has been used (e.g., James 1979, Swinscow and Krog 1979, Awasthi 1986, Stevens 1999, Clerc 2006). Therefore the delimitations of species in this group are still controversial.

It has been known that *U. rubicunda* and *U. rubrotincta* together form a monophyletic clade in the molecular phylogenetic trees of the genus *Usnea* based on internal transcribed spacer regions of nuclear ribosomal DNA (ITS rDNA) sequences (Ohmura 2002, Ohmura and Kanda 2004). However, the monophyly of each species has not been confirmed to date. Amplification of ITS rDNA sequence had been sometimes unsuccessful in these species, even though the material was fresh enough for the DNA extraction (unpublished data). It might be possible that the primers were mismatching for the annealing region of the sequence.

The purpose of this study was to examine the taxonomic distinction between *U. rubicunda* and *U. rubrotincta* based on morphological, chemical, and molecular phylogenetic data.

Materials and Methods

This study is based on 26 herbarium specimens of *Usnea rubicunda* (n = 10) and *U. rubrotincta* (n = 16) deposited in the National Museum of Nature and Science (TNS). They were collected from Japan, New Zealand, and North America between 1996 and 2007 (Table 1).

Morphological observations were made using a dissecting microscope.

Lichen substances were examined using thin layer chromatography (TLC) (Culberson and Johnson 1982). The Solvent B system (hexane: methyl tert-butyl ether: formic acid, 140:72:18) was used for all TLC analyses.

Materials for molecular phylogenetic analyses include *U. rubicunda* and *U. rubrotincta* as well as *U. glabrescens* Vain., *U. sphacelata* R. Br. and *U. subfloridana* Stirt. as out groups. The out group taxa are selected from sections *Usnea* (*U. glabrescens* and *U. subfloridana*) and *Neuropogon* (*U. sphacelata*). These sections are the sister groups of section *Ceratinae* which includes *U. rubicunda* and *U. rubrotincta* (see Ohmura and Kanda 2004). Six sequences were retrieved from DDBJ/EMBL/Genbank, which were studied by the author (Ohmura 2002). The collection data and DDBJ/EMBL/GenBank accession numbers of the ITS rDNA sequences used in this study are shown in Table 1.

DNA extractions from 5–10 mg of lichen thalli were made using a FastDNA SPIN Kit (Qbiogene) following the method of Ohmura et al. (2006).

PCR amplification of the ITS rDNA region was performed using the primer set of ITS1F (Gardes and Bruns 1993) as the 5' primer and LR1 (Vilgalys and Hester 1990) as the 3' primer. In some cases, when no PCR product appeared using ITS1F/LR1 primer pair, newly designed primers,

Table 1. Collection data and DDBJ/EMBL/GenBank accession numbers of the ITS rDNA sequences used in this study

Species	specimen number	locality and collection date	Accession number
U. rubicunda	HK42567B	Japan, Honshu, Pref. Nagano, Shimoina-gun, Minamishinano-mura, Shimonakane, 550 m, on *Cryptomeria japonica*, 5.10.2000	*AB244611
	JL478	USA, North Carolina, Jackson Co., SE of Cashiers, Chimneytop Mountain, 1450 m, on trees, 15.7.2002	AB244612
	JL562	USA, North Carolina, Jackson Co., Nantahaia National Forest, Whiteside Mountain, on a fallen branch, 23.11.2002	AB244613
	YO3114	Japan, Honshu, Pref. Nagano, Hase-mura, Todai, 1040 m, on *Pinus densiflora*, 27.6.1997	**AB051659
	YO4864	Japan, Honshu, Pref. Hiroshima, Kochi-cho, Chikurin-ji Temple, 530 m, on *Cryptomeria japonica*, 24.3.2002	AB244611
	YO5800	Japan, Honshu, Pref. Nara, Mt. Yoshino, Yoshino-Jingu Shrine, 270 m, on *Prunus yedoensis*, 29.11.2005	*AB051659
	YO5825	Japan, Honshu, Pref. Nara, Mt. Yoshino, Shimosenbon, 310 m, on *Prunus yedoensis*, 29.11.2005	*AB244611
	YO5826A	Ditto	*AB244611
	YO5826B	Ditto	AB368487
	YO5826C	Ditto	*AB244611
U. rubrotincta	DW7783	New Zealand, North Island, Wellington, Johnston Hill Reserve, 300 m, on *Berberis* sp., 10.9.2005	*AB051660
	DW7784A	Ditto	*AB051660
	HK42595	Japan, Honshu, Pref. Nagano, Shimoina-gun, Minamishinano-mura, Shimonakane, 550 m, on *Cryptomeria japonica*, 5.10.2000	*AB051660
	YO2761	Japan, Hokkaido, Prov. Kushiro, Akkeshi-gun, Akkeshi-cho, 100 m, on *Abies sachalinensis*, 3.9.1996	AB368488
	YO2841	Japan, Honshu, Pref. Aichi, Minamishitara-gun, Horai-cho, Kadoya, Mt. Horaiji, 340 m, on *Prunus* sp., 5.12.1996	*AB051660
	YO2910	Japan, Honshu, Pref. Nagano, Kawakami-mura, ca. 4 km ESE of Azusayama, 1400–1500 m, on *Larix kaempferi*, 10.12.1996	**AB051660
	YO3057	Japan, Kyushu, Pref. Ohita, Shonai-cho, en route from Hanamure to Oike, ca. 900 m, on *Quercus acutissima*, 5.3.1997	**AB051661
	YO4405	Japan, Honshu, Pref. Yamanashi, Minamitsuru-gun, Asahigaoka, Tokyo University Forest, 1500 m, on *Catalpa ovata*, 8.5.1998	AB368489
	YO4842	Japan, Kyushu, Pref. Miyazaki, Ebino-city, Shiratori Shrine, 720 m, on *Prunus* sp., 17.12.2001	*AB051661
	YO5330	Japan, Honshu, Pref. Yamanashi, Kamikuishiki-mura, Aokigahara-marubi, ca. 1000 m, on *Pinus densiflora*, 4.5.2003	*AB051661
	YO5335	Japan, Kyushu, Pref. Miyazaki, Kobayashi-city, Kiuraki, ca. 400 m, on *Prunus yedoensis*, 22.6.2003	*AB051660
	YO5827A	Japan, Honshu. Pref. Nara, Mt. Yoshino, Shimosenbon, 310 m, on *Prunus yedoensis*, 29.11.2005	*AB051661
	YO5827B	Ditto	*AB051661
	YO5827C	Ditto	*AB051660
	YO5827D	Ditto	*AB051661
	YO5970	Japan, Honshu, Pref. Yamanashi, Kamikuishiki-mura, Aokigahara-marubi, 1000 m, on *Pinus densiflora*, 3.5.2007	*AB051661
U. glabrescens	YO3824B	Japan, Honshu, Pref. Nagano, Chino-city, Yatsugatake Mts., 1740–1800m, on *Salix* sp., 29.8.1997	*AB051639
U. sphacelata	NIPR F564	Antarctica, Rundvågskollane, Sôya Coast, 17.2.1982	***AB103542
U. subfloridana	YO2879	Japan, Honshu, Pref. Nagano, Minamisaku-gun, 1860–2030 m, on *Betula ermanii*, 9.12.1996	**AB051662

Abbreviations in specimen number: DW = D. M. Wright, HK = H. Kashiwadani, JL = J. C. Lendemer, NIPR = National Institute of Polar Research, YO = Y. Ohmura. *The ITS rDNA sequence is the same with that of the accession number. **The sequence was studied in Ohmura (2002); and *** in Ohmura and Kanda (2004). All voucher specimens are preserved in TNS.

Fig. 1. Primer map for ITS rDNA region.

USITS1-F (5'-ACC TGC GGA AGG ATC ATT AC-3') as the 5' primer and USITS2-R (5'-CTG TTG GTT TCT TTT CCT-3') as the 3' primer, were used for the PCR (Fig. 1). The protocols and the sequencing, except the primers, were the same as Ohmura et al. (2006).

Sequence alignment of ITS rDNA region (including ITS1, 5.8S rDNA, and ITS2) was performed using ClustalW ver. 1.83 (Thompson et al. 1994). After removing sites with gaps, missing data and ambiguous data, the resulting alignment of 489 sites was used for the molecular phylogenetic analyses.

Molecular phylogenetic analyses were performed with maximum parsimony (MP) method (Fitch 1971) using MEGA ver. 4.0 (Tamura et al. 2007), maximum likelihood (ML) method (Felsenstein 1981) using PAUP* ver. 4.0b10 (Swofford 2002) and Bayesian Markov chain Monte Carlo (B/MCMC) method (Larget and Simon 1999) using MrBayes ver. 3.0b4 (Huelsenbeck and Ronquist 2001). The MP tree was determined using a max-mini branch-and-bound algorithm. For the ML and Bayesian analyses of the data set, TrNef + G model was selected by a hierarchical likelihood ratio test using Modeltest 3.7 (Posada and Crandall 1998): substitution rate matrix A–C = 1.0000, A–G = 1.8934, A–T = 1.0000, C–G = 1.0000, C–T = 8.0655, and G–T = 1.0000; gamma distribution shape parameter α = 0.0926; and proportion of invariable sites I = 0. ML analysis was started with a Neighbor-Joining tree and subsequent heuristic search using the tree bisection-reconnection branch-swapping algorithm. All other parameters were as the default settings of PAUP. Bootstrap analysis (Felsenstein 1985) was performed 1,000 times for the MP and 100 times for ML. Posterior probabilities were approximated by sampling trees using a Markov chain Monte Carlo (MCMC) method for Bayesian tree. One out of every 100 trees was sampled for a total of 2,000,000 generations with DNA substitution parameters estimated during the search. The first 4,000 trees from 20,000 sampled trees were discarded to avoid trees that might have been sampled prior to convergence of the Markov chains.

Results

Morphological features of the examined specimens used for DNA analyses coincided well with the description of *Usnea rubicunda* and *U. rubrotincta* made by Ohmura (2001). They are mainly distinguished by the presence or absence of elongated terminal and subterminal branches and soralia on fibrils (Fig. 2). All examined specimens of *U. rubicunda* contain usnic, norstictic, menegazziaic, and stictic acids (stictic acid strain), and those of *U. rubrotincta* contain usnic, norstictic, and salazinic acids (salazinic acid strain) as major substances detected by TLC analyses.

Among 26 samples of *U. rubicunda* and *U. rubrotincta*, PCR products of five samples (Ohmura 2761, 2841 and 4405 and Kashiwadani 42595 and 42567B) were amplified by newly designed primers USITS1-F/USITS2-R. The PCR product could not be obtained by ITS1F/LR1 for the samples. ITS rDNA sequences of *U. rubicunda* and *U. rubrotincta* were 493 and 493–496 bp long respectively. They included ITS1, 5.8S rDNA, and ITS2. After removing alignment sites with gaps, 489 sequence sites were used for phylogenetic analyses. From the operational taxonomic units (OTUs) having the same sequence, only a representative OTU was used for the analyses (see Table 1, Fig.

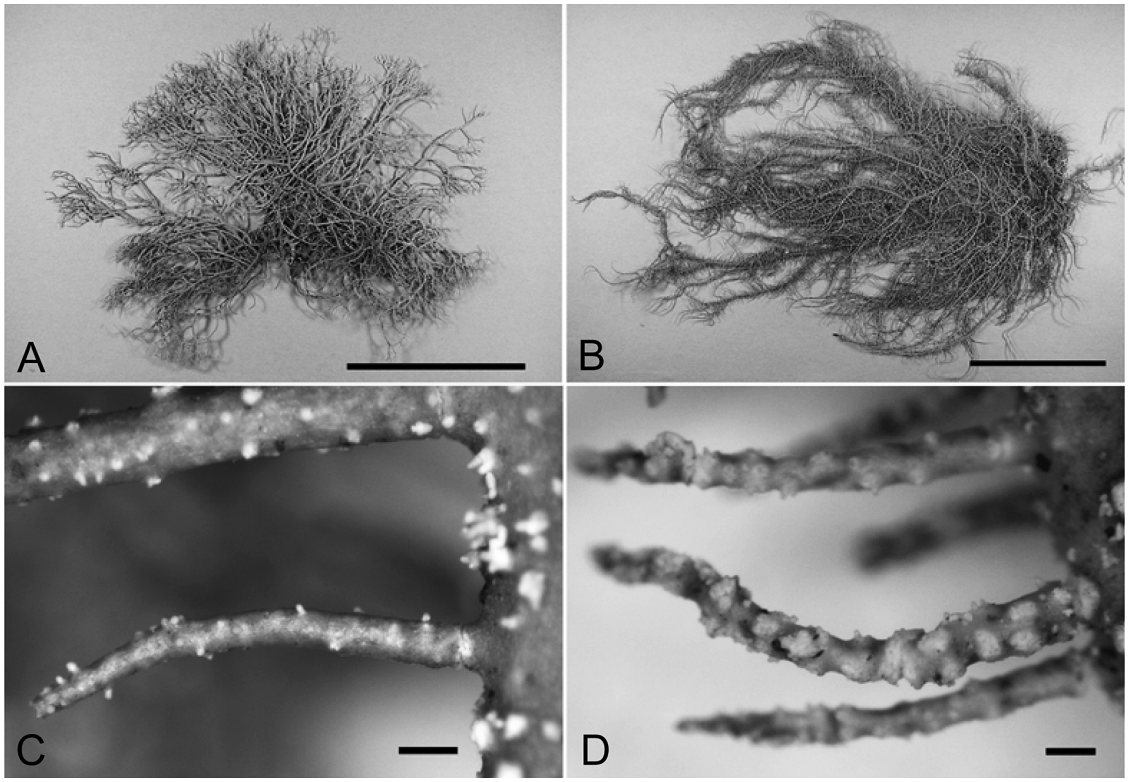

Fig. 2. Morphology of *Usnea rubicunda* and *U. rubrotincta*. A. Thallus of *U. rubicunda* with non-elongated terminal and subterminal branches. B. Thallus of *U. rubrotincta* with elongated terminal and subterminal branches. C. Fibril of *U. rubicunda* with few punctiform soralia. D. Fibril of *U. rubrotincta* with sinuose soralia. A, C. Kashiwadani 42567B. B. Kashiwadani 42595. D. Ohmura 5335. Scale: A, B = 5 cm; C, D = 200 μm.

3). The processed alignment included 55 variable sites and 34 parsimony-informative sites.

The ML tree based on the *Usnea* sequences is shown in Fig. 3. The tree was rooted by *U. glabrescens*, *U. sphacelata* and *U. subfloridana*. The OTUs of *U. rubicunda* and *U. rubrotincta* formed monophyletic clades respectively. The support values for the branches are 80% (MP), 70% (ML) and 0.97 (B/MCMC) for *U. rubicunda* and 67% (MP), 77% (ML) and 0.90 (B/MCMC) for *U. rubrotincta*. Within the *U. rubicunda* clade, the OTUs of Japanese materials formed a monophyletic clade (59% in MP and ML; 0.68 in B/MCMC). The OTUs of North American materials also formed a monophyletic clade (54% in MP; 67% in ML; 0.87 in B/MCMC). Within the *U. rubrotincta* clade, two distinct clades were formed. But no geographical differences were found between the clades.

Discussion

Morphological features of examined specimens coincided well with the description of *Usnea rubicunda* and *U. rubrotincta*. The examined specimens of each species were uniform in chemistry. They represented the stictic acid strain of *U. rubicunda* and the salazinic acid strain of *U. rubrotincta*. Using these materials, molecular phylogenetic

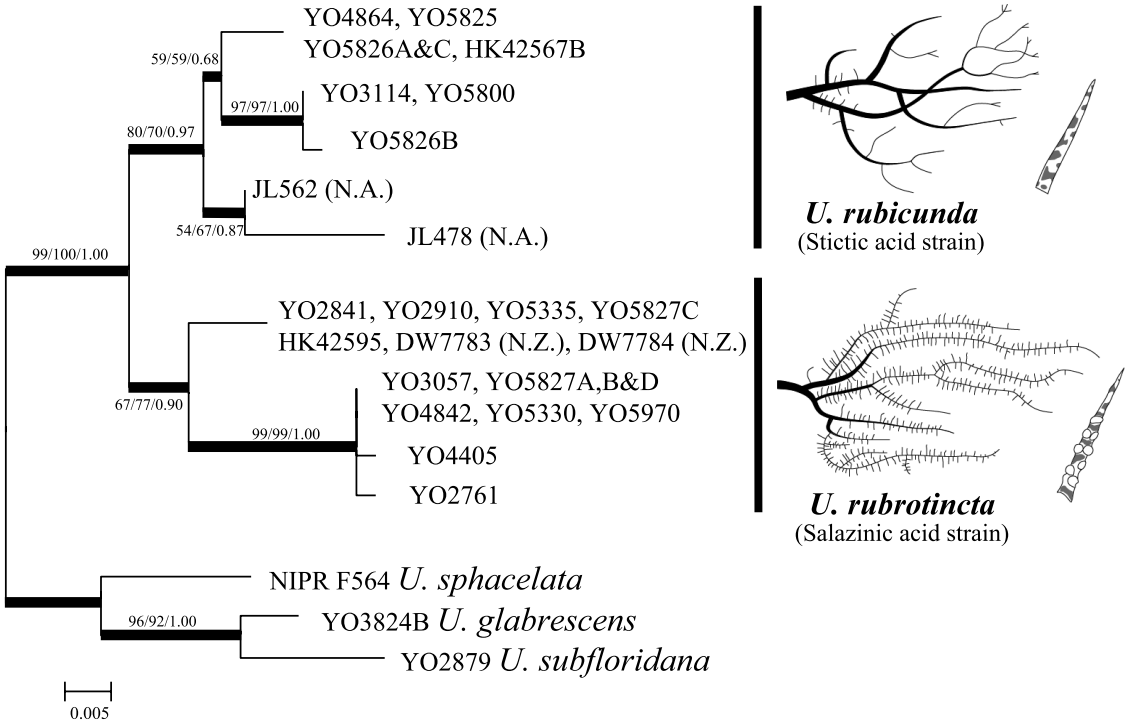

Fig. 3. Molecular phylogenetic tree of *Usnea rubicunda* and *U. rubrotincta* based on ITS rDNA sequences. The tree was constructed by ML method, and reliability of each branch was tested by MP, ML and Bayesian methods. Numbers on thicker branches indicate bootstrap values of MP (left) and ML (middle), and B/MCMC posterior probabilities (right) (they are shown only when $\geq$50% and $\geq$0.50 simultaneously). Specimen numbers are given as OTU names (see Table 1). *Usnea glabrescens*, *U. sphacelata* and *U. subfloridana* are used as out groups. These specimens of *U. rubicunda* and *U. rubrotincta* were collected from Japan unless otherwise indicated (N.A. = North America; N.Z. = New Zealand). Illustrations indicate thallus habit and fibril of *U. rubicunda* and *U. rubrotincta*.

analyses with MP, ML and B/MCMC methods based on ITS rDNA sequences showed the monophyly of both *U. rubicunda* and *U. rubrotincta*.

Usnea rubicunda (s. l.) is known to be distributed in Africa, Asia, Europe, Oceania, and North and South America (James 1979, Ohmura 2001, Clerc 2006). Since comprehensive samples from all these areas (including England, the type locality of *U. rubicunda*) and all chemical strains could not be included in this study, the molecular phylogenetic tree is preliminary. However, the molecular data are consistent with the morphological differences which have been observed in this group. Therefore, *U. rubicunda* and *U. rubrotincta* are retained as distinct species in this study. The morphological and chemical differences between these species are discussed below from the taxonomic point of view.

The most important diagnostic feature for these species is the elongation or non-elongation of terminal and subterminal branches. Branch elongation is observed in *U. rubrotincta* but never in *U. rubicunda* (Figs. 2A, B). This feature is also seen in several other *Usnea* species such as *U. angulata* Ach., *U. longissima* Ach., *U. pectinata* Taylor, and *U. trichodeoides*

Vain., and considered to be an important taxonomic characteristic in the genus (Ohmura 2001). Numerous fibrils, growing perpendicularly on terminal and subterminal branches, are usually observed in *U. rubrotincta*, whereas they are usually few to sparse in *U. rubicunda*. But the amount of fibrils in these species occasionally varies. Such variation is also known in, e.g., *U. dasaea* Strit. and *U. nidifica* Taylor (Clerc and Herrera-Campos 1997, Ohmura 2001). Therefore, this characteristic should be carefully used for distinguishing species. Soralia of *U. rubicunda* are usually absent on fibrils. If present, they are few to sparse and punctiform in shape (Fig. 2C). Soralia of *U. rubrotincta* are usually present on fibrils. When they are crowded on a fibril or lateral branch, they may become sinuose in shape (Fig. 2D). The sinuose soralia may be formed by the synergic pressure of crowded soralia. Soralia of each species seem to be developed mainly from eroded papillae on lateral or main branches in *U. rubicunda* and scars of detached fibrils or lateral branches in *U. rubrotincta*. However, both origins are more or less involved in each species. Therefore, the soralia origins may have less taxonomic value for distinguishing between species.

The chemical features in *U. rubicunda* and *U. rubrotincta* are useful for identification because the frequency of the minor strain in each species is less than 1% within eastern Asia specimens (see Ohmura 2001). The rare occurrences of those chemical strains are apparently unusual for these species. Discovery of a chemically chimeric thallus which is morphologically *U. rubrotincta* (Ohmura 2001) suggests the possibility of fusion or introgressive hybridization between different species in the genus *Usnea*. These phenomena may also possibly occur between *U. rubicunda* and *U. rubrotincta* that are sometimes growing together within the same habitat. Such phenomena are known in several other lichens (e.g., *Cladonia*, *Rhizoplaca* and *Stereocaulon*), and the hybrid may show polymorphic or intermediate phenotypes in morphology and/or chemistry (Meyer et al. 1981, Culberson et al. 1988, Fahselt 1991).

The Japanese and North American materials of *U. rubicunda* weakly form monophyletic clades respectively. Regarding the North American materials of *U. rubicunda*, Lendemer (2004) emphasized the presence of *U. pensylvanica* Motyka which should be distinguished from *U. rubicunda*. According to him, this species is characterized by the thinner cortex, the smaller and inconspicuously elevated soralia, and the duller colored red pigmentation than those of *U. rubicunda*. However, the type specimen of *U. pensylvanica* fit well within the range of *U. rubicunda* (Ohmura 2001, Clerc 2006). If the taxon (*sensu* Lendemer 2004) is a distinct species, it may be another species having red pigment in the cortex besides *U. rubicunda* and *U. rubrotincta*. Further taxonomic studies are needed to elucidate the identity of the taxon using detailed morphological and molecular phylogenetic analyses. No geographical difference was observed between the subclades of the *U. rubrotincta* clade.

The primer set ITS1F/LR1 could not amplify ITS rDNA sequence from some specimens of *U. rubicunda* and *U. rubrotincta*. However, the newly designed primer set, USITS1F/USITS2R, could successfully amplify the sequence. PCR product of ITS rDNA region in *Usnea* species sometimes contains long insertion in 18S rDNA (Ohmura 2002). The PCR reaction using ITS1F might be disturbed by the insertion.

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大村嘉人：**アカヒゲゴケおよびアカサルオガセ（ウメノキゴケ科，地衣類）の分類と分子系統**

皮層に赤色色素を持つアカヒゲゴケ *Usnea rubicunda* とアカサルオガセ *U. rubrotincta* は，伸長する末端枝の有無，フィブリルの量，ソラリアの形態などによって区別されるが，これらを同一種内の形態変異とする意見もある．両種について，ITS rDNA 配列に基づく最節約法，最尤法およびベイズ法による分子系統解析を行った結果，それぞれ単系統群を形成したことから，独立種として扱うことが支持された．なお，PCR 産物が通常のプライマーセット（ITS1F/LR1）によって得られなかったサンプルについては，新たに設計したプライマー（USITS1-F/USITS2-R）によって ITS rDNA 配列を得ることができた．

（国立科学博物館植物研究部）